Sodium- and Chloride-Dependent, Cocaine-Sensitive, High-Affinity Binding of Nisoxetine to the Human Placental Norepinephrine Transporter†

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ABSTRACT: Maternal-facing brush border membranes prepared from normal term human placentas possess the norepinephrine transporter. We investigated the interaction of nisoxetine with the human norepinephrine transporter by examining the binding of this ligand to the placental brush border membranes. Scatchard analysis revealed that nisoxetine bound with high affinity to a single class of binding sites in the membranes (dissociation constant = 13.8 ± 0.4 nM). This value obtained from equilibrium experiments matched the value (11.2 nM) which was calculated using the association and dissociation rate constants. The maximal binding capacity (B_{max}) was 5.1 ± 0.1 pmol/mg of protein. The binding exhibited an absolute requirement for Na^+ as well as Cl^-. Presence of these ions enhanced the binding affinity without affecting B_{max}. Kinetic analyses revealed that the coupling ratio of Na^+/nisoxetine was 2, whereas the coupling ratio of Cl^-/nisoxetine was 1. The binding was most potently inhibited by the ligands of the norepinephrine transporter (desipramine and nomifensine). The ligands of the serotonin transporter (imipramine, paroxetine, and fluoxetine) showed intermediate inhibitory potencies, whereas the ligands of the dopamine transporter (bupropion and GBR 12909) were the least potent. Among the monoamines, dopamine was the most potent inhibitor, followed by norepinephrine and serotonin. Though both cocaine and its analog RTI-55 were powerful inhibitors of the binding, RTI-55 was ≈150 times more effective than cocaine. The inhibition of binding by norepinephrine, cocaine, and RTI-55 was competitive. Uptake of norepinephrine measured in membrane vesicles was found to be inhibited by treatment of the vesicles with phenylarsine oxide, a reagent specific for vicinal dithiol groups. The transporter was protected to a significant extent from this inhibition if norepinephrine or nisoxetine was present during treatment with the reagent, indicating that the binding sites on the transporter for norepinephrine and nisoxetine are either identical or considerably overlap with each other. The binding of nisoxetine to the membranes was pH dependent with a pK_a of 5.7. Analysis of the influence of pH on the kinetic parameters for nisoxetine binding with respect to the dependence of binding on the concentration of Na^+, Cl^-, and nisoxetine showed that H^+ decreased the affinity of the transporter for Na^+, Cl^-, and nisoxetine, but did not influence B_{max}, nor did it alter the Na^+/nisoxetine and Cl^-/nisoxetine coupling ratios. K^+ showed a small, but significant, stimulatory effect on the binding. Zn^{2+} at concentrations ≤100 μM significantly stimulated the binding but caused inhibition at higher concentrations. This detailed report on the characteristics of the binding of nisoxetine to the norepinephrine transpoprter from a tissue of human origin show that nisoxetine can be used as a specifc, high-affinity ligand in studies involving the human norepinephrine transporter.

Our current understanding of the nature of the three biogenic amine transporters, namely, the serotonin, dopamine, and norepinephrine transporters, has been greatly facilitated by the discovery of compounds which act as high-affinity ligands for these transporters. In most instances, these ligands specifically bind to the substrate-binding site of the transporters and block the transporter function. Interestingly, even though these ligands interact with the substrate-binding site, they are not transportable substrates. Thus, the transporters act very much like receptors for these ligands. Due to their ability to interfere with the function of the biogenic amine transporters, these ligands are receiving increasing attention for their potential in the treatment of various clinical disorders involving the monoaminergic pathways. In addition, availability of many of these ligands in radiolabeled form facilitates their use as specific probes to delineate the nature of the substrate-binding sites of the transporters and also aids in the isolation of the transport proteins by offering convenient means via binding assays to monitor the presence of the proteins during solubilization and purification stages.

The biochemical, pharmacological, and clinical uses of these ligands are best exemplified in the case of the serotonin transporter with its specific ligands, such as fluoxetine, paroxetine, imipramine, and citalopram (Marcusson & Ross, 1990; Graham & Langer, 1992). The ligands of similar use for the dopamine transporter include GBR12909, GBR12935, nomifensine, and BTCP[1] (Horn, 1990; Graham & Langer, 1992). However, a comparatively smaller number of such suitable ligands are currently available for characterization of the norepinephrine transporter. Several investigators (Backstrom *et al.*, 1989; Bonisch & Harder, 1986; Lee & Snyder, 1981; Lee *et al.*, 1982; Schomig & Bonisch, 1986) have employed desipramine as the probe for investigating the norepinephrine transporter, but this ligand is less than ideal

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[1] Abbreviations: BTCP, *N*-[1-(2-benzo[*b*]thiophenyl)cyclohexyl]piperidine; RTI-55, 2β-carbomethoxy-3β-(4-iodophenyl)tropane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; cyclo(His-Pro), histidylproline diketopiperazine.

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because of its interaction with additional sites that are not related to the norepinephrine transporter (Lee & Snyder, 1981; Lee *et al.*, 1982; Backstrom *et al.* 1989). Mazindol and nomifensine interact not only with the norepinephrine transporter but also with the dopamine transporter (Javitch *et al.*, 1984; Dubocovich & Zahniser, 1985). Nisoxetine [*N*-methyl-3-(*o*-methoxyphenoxy)-3-phenylpropylamine] appears to be a better ligand than the above-mentioned ligands due to its high specificity toward the norepinephrine transporter and its lack of affinity toward various receptors (Wong & Bymaster, 1976; Wong *et al.*, 1982). Only recently, however, have studies been initiated with radiolabeled nisoxetine to characterize the binding of this ligand to the norepinephrine transporter (Tejani-Butt, 1992; Tejani-Butt *et al.*, 1990). All of these studies have been done only in the rat. Even though [^{3}H]-nisoxetine has been successfully used to quantitate norepinephrine uptake sites in the human brain (Tejani-Butt & Ordway, 1992), to date there is no information available on the characteristics of interaction between this ligand and the human norepinephrine transporter. In this paper, we report for the first time a detailed and systematic analysis of binding of [^{3}H]nisoxetine to the human norepinephrine transporter.

The syncytiotrophoblast of the human placenta expresses two of the biogenic amine transporters, the serotonin transporter and the norepinephrine transporter (Ganapathy *et al.*, 1993). The maternal-facing brush border membrane vesicles isolated from normal term human placentas possess both of these transporters (Balkovetz *et al.*, 1989; Ramamoorthy *et al.*, 1992, 1993). These membranes have been previously employed to investigate the interaction of [^{3}H]paroxetine with the serotonin transporter (Cool *et al.*, 1990a). In the present study, the characteristics of the interaction of [^{3}H]nisoxetine with the norepinephrine transporter have been investigated using a similar experimental approach.

MATERIALS AND METHODS

Materials. [*N-methyl*-^{3}H]Nisoxetine (specific radioactivity, 8.2 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and *levo*-[7-^{3}H]norepinephrine (specific radioactivity, 11.4 Ci/mmol) was purchased from Du Pont-New England Nuclear (Boston, MA). Desipramine, imipramine, cocaine, dopamine, norepinephrine, serotonin, and cyclo(His-Pro) were obtained from Sigma (St. Louis, MO). Bupropion, GBR12909, and nomifensine were from Research Biochemicals, Inc. (Natick, MA). Phenylarsine oxide was obtained from Aldrich Chem. Co. (Milwaukee, WI). Unlabeled nisoxetine and fluoxetine were generous gifts from Eli Lilly Pharmaceuticals (Indianapolis, IN). Paroxetine was a gift from Beecham Pharmaceuticals (Betchworth, Surrey, UK). RTI-55, an analog of cocaine, was kindly provided by Dr. F. I. Carroll, Research Triangle Institute (Research Triangle Park, NC). All other chemicals used were of analytical grade.

Preparation of Human Placental Brush Border Membranes. Maternal-facing brush border membranes were prepared from normal term human placentas by the Mg^{2+}-aggregation method as described previously (Balkovetz *et al.*, 1986; Kulanthaivel *et al.*, 1990a). The final membrane preparations were suspended in three different buffers as required by individual experiments: 50 mM Tris-HCl, pH 7.4, containing 300 mM NaCl and 5 mM KCl; 50 mM Tris-HCl, pH 7.4, containing 300 mM mannitol; 10 mM Hepes/Tris, pH 7.4, containing 300 mM mannitol. Protein concentration in final preparations was adjusted to 3 mg/mL and the membranes were then stored in liquid N_2 in small aliquots until used. Up to 4 weeks in storage, no change in [^{3}H]nisoxetine binding was detected.

Nisoxetine Binding Assay. Binding of [^{3}H]nisoxetine to placental brush border membranes was assayed as described by Tejani-Butt *et al.* (1990), with minor modifications. The reaction between brush border membranes (150 μg of protein) and [^{3}H]nisoxetine in a total volume of 0.25 mL was carried out at room temperature (22 °C) unless indicated otherwise. A 60-min incubation was used for determination of equilibrium binding in all experiments except those involving cocaine and RTI-55, in which case a 30-min incubation was employed, due to the unstable nature of these compounds. The binding buffer was 50 mM Tris-HCl, pH 7.4, containing 300 mM NaCl and 5 mM KCl, unless indicated otherwise. Binding was terminated by addition of 3 mL of ice-cold binding buffer, followed by rapid filtration of the reaction mixture on a Whatman GF/F glass fiber filter which had been presoaked in 0.3% polyethylenimine. The filter was washed with 3 × 5 mL of ice-cold binding buffer and the radioactivity associated with the filter was then determined by scintillation spectrometry. Specific binding was routinely calculated by subtracting the binding measured in the presence of 10 μM unlabeled nisoxetine from the total binding. Nonspecific binding never exceeded 5% of total binding when the concentration of [^{3}H]nisoxetine was $\leq$5 nM.

Uptake Measurement in Membrane Vesicles. Uptake of radiolabeled norepinephrine into placental brush border membrane vesicles was determined as described previously (Ramamoorthy *et al.*, 1993). Membrane vesicles suspended in 10 mM Hepes/Tris buffer, pH 7.5, containing 75 mM potassium gluconate and 150 mM mannitol were treated with or without 1 mM phenylarsine oxide for 1 h at room temperature (Kulanthaival *et al.*, 1990b). When present during this treatment, the concentration of norepinephrine was 0.2 mM and that of nisoxetine was 10 μM. Following the treatment, the reagents were removed by dilution and centrifugation. The membrane vesicles were finally suspended in a small volume of the treatment buffer and employed in uptake measurements. The composition of the uptake medium was 10 mM Hepes/Tris (pH 7.5) and 150 mM NaCl. Uptake rates of norepinephrine (0.2 μM) were measured using a 15-s incubation.

Data Analysis. Binding measurements were performed in duplicate or triplicate, using two to three different membrane preparations. The results are presented as means ± SE for these replicate determinations. Kinetic analyses were conducted using a commercially available computer program *Fig. P* 6.0 (BioSoft, Cambridge, UK).

RESULTS

Influence of Incubation Time and Temperature on the Binding of Nisoxetine to Human Placental Brush Border Membranes. Binding of nisoxetine (concentration, 2nM) to placental brush border membranes was measured at 4 °C and at room temperature (22 °C), and Figure 1 shows the time course of binding at these two temperatures. Binding was considerably slower at 4 °C than at 22 °C. Maximal binding occurred within 15 min of incubation at 22 °C, whereas a 4-h incubation was required to obtain maximal binding at 4 °C. Therefore, in subsequent experiments equilibrium binding was measured routinely by conducting binding experiments at 22 °C with a 60-min incubation.

Requirements for Na^+ and Cl^- for Nisoxetine Binding. Transport of dopamine as well as norepinephrine via the norepinephrine transporter in human placental brush border

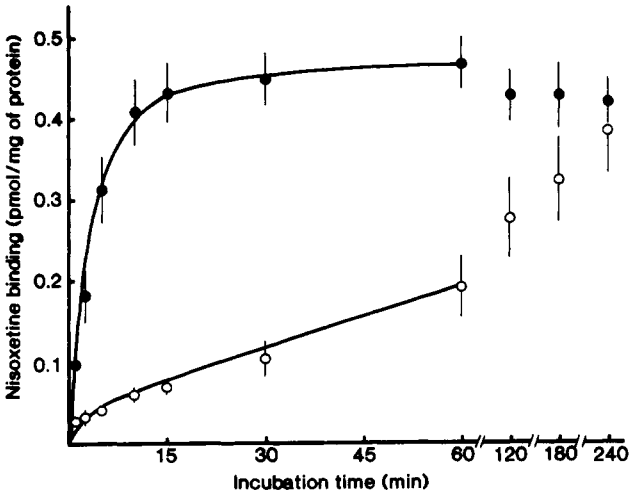

FIGURE 1: Influence of temperature and incubation time on nisoxetine binding. Human placental brush border membranes (200 μg protein) were incubated with 2 nM [^{3}H]nisoxetine at either 4 °C (○) or room temperature (22 °C, ●) for varying time periods in 50 mM Tris-HCl buffer, pH 7.4, containing 300 mM NaCl and 5 mM KCl. Binding was determined as described under Materials and Methods. Values are means ± SE for four determinations. When not shown, SE was within the symbol.

membrane vesicles shows an absolute requirement for Na^+ and Cl^- (Ramamoorthy *et al.*, 1992, 1993). Therefore, we investigated the ionic requirements for equilibrium binding of nisoxetine (2 nM) to placental brush border membranes. Binding of nisoxetine was maximal only when the binding buffer contained Na^+ as well as Cl^- and removal of either Na^+ or Cl^- from the assay medium completely abolished the binding (data not shown). Replacement of NaCl with mannitol also resulted in almost total inhibition of nisoxetine binding. These results show that the binding of nisoxetine to placental brush border membranes is Na^+ and Cl^- dependent.

Dependence of Nisoxetine Binding on the Concentration of Na^+. Equilibrium binding of nisoxetine (3 nM) to placental brush border membranes was measured at increasing concentrations of Na^+ (0–300 mM), but at a fixed concentration of Cl^- (300 mM). Figure 2 shows the dependence of binding on Na^+ concentration. The presence of Na^+ stimulated the binding in a concentration-dependent manner. The relationship between the binding and the Na^+ concentration was sigmoidal, suggesting that the coupling ratio of Na^+/nisoxetine is greater than one. The data were analyzed using the Hill-type equation

$$B = \frac{B_M[Na^+]^n}{K^n_{0.5} + [Na^+]^n}$$

where B is nisoxetine binding, B_M is maximal nisoxetine binding, $[Na^+]$ is the concentration of Na^+, $K_{0.5}$ is the concentration of Na^+ necessary to elicit 50% of the maximal effect, and n is the number of Na^+ ions involved in the binding process. This analysis gave a value of 251 ± 12 mM for $K_{0.5}$ and 2.08 ± 0.06 for n. The linearity (r = –0.98) of the Hill-type plot (Figure 2, inset) with n = 2, i.e., B vs $B/[Na^+]^2$, supports the conclusion that the coupling ratio of Na^+/nisoxetine is 2.

Dependence of Nisoxetine Binding on the Concentration of Cl^-. Similar experiments on the dependence of equilibrium binding of nisoxetine (3 nM) on the concentration of Cl^- done in the presence of a fixed concentration of Na^+ revealed that the relationship in the case of Cl^- was not sigmoidal but instead hyperbolic (Figure 3). These results suggest that the coupling

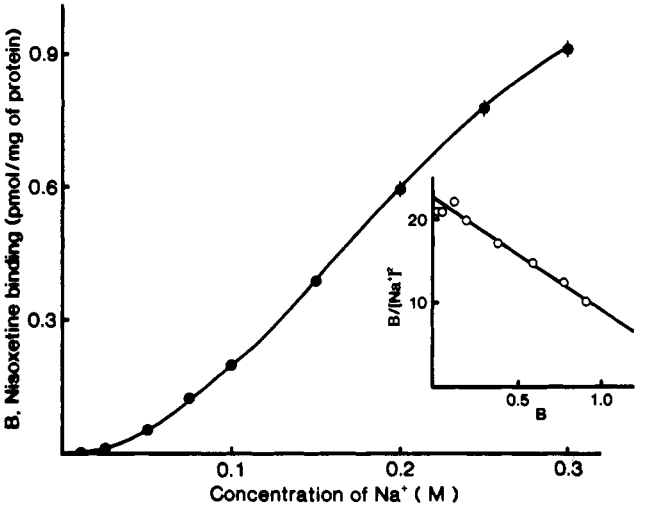

FIGURE 2: Na^+ dependence of nisoxetine binding. Equilibrium binding of 3 nM [^{3}H]nisoxetine to human placental brush border membranes was assayed at room temperature with a 60-min incubation. Binding buffers contained varying concentrations of Na^+ (0–300 mM) and a fixed concentration of Cl^- (300 mM). These buffers were prepared by appropriately mixing two solutions containing 400 mM NaCl or 400 mM LiCl, buffered with 10 mM Hepes/Tris, pH 7.4. Only the values for Na^+-dependent binding (binding in the presence of Na^+ minus binding in the absence of Na^+) were used in the data analysis. Values are means ± SE for four determinations. When not shown, SE was within the symbol. Inset: Hill-type plot (B vs $B/[Na^+]^n$) with a value of 2 for n (number of binding sites for Na^+).

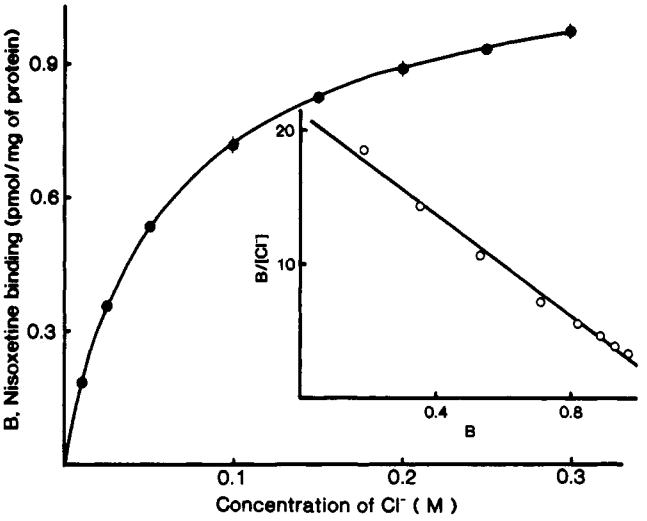

FIGURE 3: Cl^- dependence of nisoxetine binding. Equilibrium binding of 3 nM [^{3}H]nisoxetine to human placental brush border membranes was assayed at room temperature with a 60-min incubation. Binding buffers contained varying concentrations of Cl^- (0–300 mM) and a fixed concentration of Na^+ (300 mM). These buffers were prepared by appropriately mixing two solutions containing 400 mM NaCl or 400 mM sodium gluconate, buffered with 10 mM Hepes/Tris, pH 7.4. Only the values for Cl^--dependent binding (binding in the presence of Cl^- minus binding in the absence of Cl^-) were used in the data analysis. Values are means ± SE for four determinations. When not shown, SE was within the symbol. Inset: Hill-type plot (B vs $B/[Cl^-]^n$) with a value of 1 for n (number of binding sites for Cl^-).

ratio of Cl^-/nisoxetine is one. Analysis of the data using the Hill-type equation as described earlier for Na^+ gave a value of 0.90 ± 0.01 for n (the number of Cl^- ions involved in the binding process) and 68 ± 2 mM for $K_{0.5}$ (the concentration of Cl^- necessary to produce 50% of the maximal effect). The linearity (r = –0.99) of the Hill-type plot (Figure 3, inset) with n = 1, i.e., B vs $B/[Cl^-]$, supports the Cl^-/nisoxetine coupling ratio of 1.

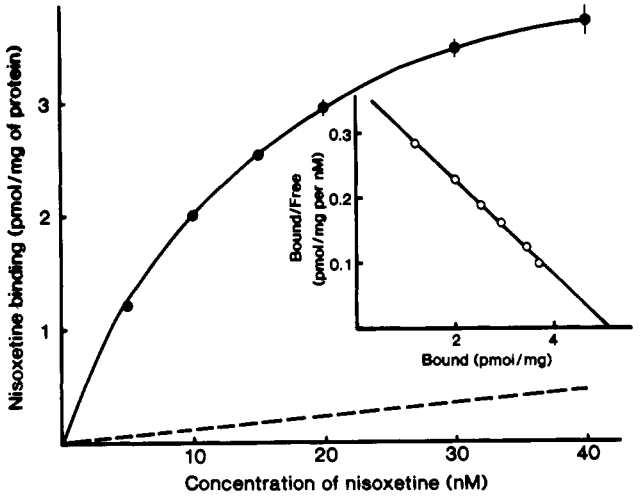

FIGURE 4: Saturation analysis of nisoxetine binding. Equilibrium binding of nisoxetine to human placental brush border membranes was assayed at room temperature with a 60-min incubation. Binding buffer was 50 mM Tris-HCl, pH 7.4, containing 300 mM NaCl and 5 mM KCl. Nisoxetine concentration was varied over a range of 5–40 nM. At each concentration, [^{3}H]nisoxetine was 5 nM and the remainder was unlabeled nisoxetine. Nonspecific binding was determined from the binding of radiolabel measured in the presence of 10 μM unlabeled nisoxetine (dotted line). Values are means ± SE for four determinations. When not shown, SE was within the symbol. Inset: Scatchard plot for specific binding.

Scatchard Analysis of Nisoxetine Binding. Figure 4 shows the relationship between equilibrium binding and nisoxetine concentration. Total binding measured over a nisoxetine concentration of 5–40 nM showed saturability. In contrast, the nonspecific binding was not saturable. Scatchard analysis of specific binding (Figure 4, inset) gave a value of 13.8 ± 0.4 nM for K_d (dissociation constant) and 5.1 ± 0.1 pmol/mg of protein for B_{max} (maximal binding capacity).

Dissociation and Association Rate Constants. We further analyzed the interaction of nisoxetine with the placental brush border membranes by determining the rate constants for the dissociation and association processes (Bennet, 1978; Cool *et al.*, 1990b). The membranes were first incubated with 2 nM of [^{3}H]nisoxetine for 1 h to reach equilibrium, following which the time course of dissociation of the radioligand was monitored by diluting the membranes in the presence of excess (10 μM) amount of unlabeled nisoxetine (Figure 5A). The dissociation was rapid, and the time required for 50% dissociation was less than 5 min. The dissociation rate constant, K_{-1}, was calculated from the equation $\ln (B_t/B_0) = -K_{-1}t$, where B_t is the binding of [^{3}H]nisoxetine at time t and B_0 is the binding of [^{3}H]-nisoxetine at time zero (i.e. at the initiation of dissociation). A plot of $\ln (B_t/B_0)$ vs t was linear (Figure 5B, $r = -0.999$) with a slope (K_{-1}) of 0.19. The association rate constant (K_{+1}) was determined from the time course of [^{3}H]nisoxetine binding at room temperature described in Figure 1. K_{+1} was calculated from the equation $\ln (B_0/(B_0 - B_t)) = ([L]K_{+1} + K_{-1})t$ where B_0 is the binding of [^{3}H]nisoxetine at equilibrium (1 h), B_t is the binding at time t and [L] is the concentration of [^{3}H]-nisoxetine in the binding assay. The plot of $\ln (B_0/(B_0 - B_t))$ vs t was linear ($r = 0.999$) with a slope of 0.223 which was equal to $[L]K_{+1} + K_{-1}$ (data not shown). By substitution of the values for [L] (2 nM) and K_{-1} (0.19), the value for K_{+1} was calculated to be 0.0165. The equilibrium dissociation constant (K_d) calculated as the ratio of K_{-1}/K_{+1} was 11.5 nM. This value matched the K_d value (13.8 nM) calculated from the equilibrium experiments.

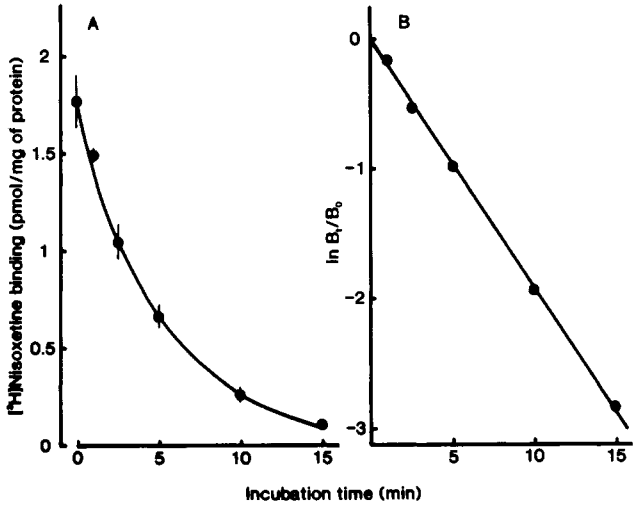

FIGURE 5: Equilibrium dissociation of [^{3}H]nisoxetine. Human placental brush border membranes were incubated with 2 nM [^{3}H]-nisoxetine in the presence of 300 mM NaCl and 5 mM KCl in 50 mM Tris-HCl buffer, pH 7.4, for 1 h at room temperature to attain equilibrium binding. Following this incubation, the membranes were diluted into the binding buffer containing 10 μM unlabeled nisoxetine and incubated for indicated different time intervals and then the amount of [^{3}H]nisoxetine still bound to the membranes was determined. Key: (A) time course of dissociation of [^{3}H]nisoxetine; (B) plot of ln (B_t/B_0) verus time where B_0 is the amount of [^{3}H]-nisoxetine associated with the membranes at equilibrium prior to the initiation of dissociation (zero time) and B_t is the amount of [^{3}H]-nisoxetine associated with the membranes at indicated time periods following the initiation of dissociation. Values are means ● SE for three determinations.

Table I: Influence of Na^+ and Cl^- on the Kinetic Parameters, Dissociation Constant (K_d) and Maximal Binding Capacity (B_{max}) for Binding of Nisoxetine[a]

[Na^+] (mM)	[Cl^-] (mM)	K_d (nM)	B_{max} (pmol/mg of protein)
300	300	1.38 ± 0.4	5.1 ± 0.1
150	300	38.7 ● 4.3	4.7 ± 0.4
300	50	25.9 ± 1.2	4.5 ± 0.2

[a] Human placental brush border membranes, suspended in 10 mM Hepes/Tris buffer, pH 7.4, containing 300 mM mannitol, were used in the binding assay. Binding buffers were 10 mM Hepes/Tris, pH 7.4, containing 500 mM NaCl and 250 mM NaCl plus 250 mM LiCl or 83.33 mM NaCl plus 416.67 mM sodium gluconate, which gave the following final concentrations during binding measurement, respectively: 300 mM Na^+ plus 300 mM Cl^-, 150 mM Na^+ plus 300 mM Cl^-, and 300 mM Na^+ plus 50 mM Cl^-. Binding was assayed over a nisoxetine concentration range of 5–40 nM (concentration of [^{3}H]nisoxetine was 5 nM and the remaining was unalbeled nisoxetine). Assay was done with a 60-min incubation at room temperature. Nonspecific binding was determined by performing the binding assay in the presence of 10 μM unlabeled nisoxetine. Kinetic constants, K_d and B_{max}, were determined by Scatchard analysis of the experimental data. Values are means ± SE for 4–16 determinations.

Influence of Na^+ and Cl^- on the Kinetic Parameters of Nisoxetine Binding. Since binding of nisoxetine showed an absolute requirement for Na^+ and Cl^-, the influence of these ions on the kinetic parameters, dissociation constant (K_d) and maximal binding capacity (B_{max}) was determined. The results are summarized in Table I. Control experiments done in the presence of 300 mM Na^+ and 300 mM Cl^- gave a value of 13.8 ± 0.4 nM for K_d and 5.1 ± 0.1 pmol/mg of protein for B_{max}. Reducing the concentration of Na^+ from 300 to 150 mM (the concentration of Cl^- was kept at 300 mM) resulted in an increase in K_d (38.7 ± 4.3 vs 13.8 ± 0.4 nM), indicating that the presence of Na^+ increases the binding affinity. There was no significant change in B_{max} whether the Na^+ concentration was 300 or 150 mM. Similarly, reducing the

concentration of Cl^- from 300 to 50 mM (the concentration of Na^+ was kept at 300 mM) also had similar effects on the binding affinity. The value for K_d increased as the concentration of Cl^- was decreased (25.9 ± 1.2 vs 13.8 ± 0.4 nM). Again, B_{max} remained largely unaffected. These results show that both Na^+ and Cl^- enhance nisoxetine binding by increasing the affinity of the binding site for its ligand.

Influence of Monoamine Uptake Inhibitors, Monoamines, and Cocaine on [^{3}H]Nisoxetine Binding. The effects of various monoamine uptake inhibitors on the binding of [^{3}H]nisoxetine (2 nM) were studied using increasing concentrations of each inhibitor. Among the compounds employed, the ligands of the norepinephrine transporter, desipramine, nomifensine, and nisoxetine, were the most potent inhibitors of [^{3}H]nisoxetine binding. The ligands of the serotonin transporter exhibited intermediate inhibitory potencies (order of potency: imipramine > paroxetine > fluoxetine), whereas the ligands of the dopamine transporter (bupropion and GBR 12909) were the least potent. When the influence of the three monoamines on the binding of nisoxetine was studied in a similar manner, it was found that dopamine was the most potent inhibitor, followed by norepinephrine and serotonin. We also investigated the effects of cocaine and its analog RTI-55 on the binding of nisoxetine. Both compounds inhibited the binding, but the potency of RTI-55 was at least two orders of magnitude greater than that of cocaine. The inhibition constants (K_i) for the above-mentioned compounds, calculated from the dose–response relationship, are given in Table II.

Influence of Norepinephrine, Cocaine, and RTI-55 on the Kinetic Parameters of Nisoxetine Binding. Since the inhibitor specificity of nisoxetine binding clearly revealed that the norepinephrine transporter in the placental brush border membranes was responsible for the observed binding, we determined the effects of norepinephrine on the kinetic parameters of nisoxetine binding (Table III). Control experiments done in the absence of norepinephrine gave a value of 13.8 ± 0.4 nM for K_d and 5.1 ± 0.1 pmol/mg of protein for B_{max}. The presence of 300 μM norepinephrine during the binding assay increased the K_d value 4.3-fold. The influence of the monoamine on B_{max} was very small (6.6 ± 0.4 vs 5.1 ± 0.1 pmol/mg of protein). These results show that the inhibition of nisoxetine binding by norepinephrine is competitive in nature, suggesting that a common site is likely involved in the binding of these two compounds.

Similar experiments with cocaine and RTI-55 revealed that the inhibition of nisoxetine binding by these compounds is also predominantly due to a decrease in the affinity of the binding site for nisoxetine. Both cocaine and RTI-55 significantly increased the value for K_d. The influence of these inhibitors on B_{max} was very small (Table III). Thus, cocaine and RTI-55 are also competitive inhibitors of nisoxetine binding.

Similarity between the Binding Sites for Norepinephrine and Nisoxetine. To determine the similarity between the chemical nature of the binding sites for norepinephrine and nisoxetine on the placental norepinephrine transporter, we studied the inactivation of the transport function of the transporter by thiol group modifying agents and assessed the ability of norepinephrine and nisoxetine to protect the transporter from the inhibition. We selected phenylarsine oxide for this purpose. This reagent is specific for vicinal dithiol groups. Treatment of placental brush border membrane vesicles with this reagent inhibited norepinephrine transport (47 ± 2% inhibition at 1 mM phenylarsine oxide), indicating that the reactive dithiol groups are essential for the transport function. However, the transporter was protected to a significant extent from the phenylarsine oxide-induced inhibition if treatment with the reagent was done in the presence of norepinephrine or nisoxetine. The presence of 0.2 mM norepinephrine and 10 μM nisoxetine during the treatment reduced the phenylarsine oxide-induced inhibition from 47 ± 2% to 21 ± 1% and 26 ± 1%, respectively. These results show that the reactive dithiol groups are located at or near the binding site(s) for both norepinephrine and nisoxetine. Norepinephrine and nisoxetine interact with the transporter reversibly, and therefore occupation of the binding site(s) by these compounds apparently renders the dithiol groups inaccessible to the modifying agent and thereby protects the groups from chemical modification. It thus appears that the binding sites for the substrate norepinephrine and the antagonist nisoxetine are either identical or exhibit a considerable steric overlap.

Influence of pH on Nisoxetine Binding. To characterize further the binding of nisoxetine to placental brush border membranes, the influence of pH on the binding was determined (data not shown). The binding was very small at pH 5.0 but increased as the pH increased. Maximal binding was observed

Table II: Inhibition Constants (K_i) for Various Drugs and Monoamines To Inhibit Nisoxetine Binding[a]

drugs or monoamines	K_i (μM)
Drugs	
desipramine	0.011 ± 0.001
RTI-55	0.055 ± 0.003
nomifensine	0.117 ± 0.008
imipramine	0.223 ± 0.009
paroxetine	0.969 ± 0.048
cocaine	8.36 ± 0.62
fluoxetine	8.57 ± 0.25
bupropion	71.39 ± 5.27
GBR 12909	176.69 ± 28.51
Monoamines	
dopamine	19.0 ± 2.4
norepinephrine	254.2 ± 17.2
serotonin	818.6 ± 37.2

[a] Inhibition constants (K_i) were calculated from the IC_{50} values (concentration necessary to cause 50% inhibition) obtained from the dose-response relationship for each compound according to the method of Cheng & Prusoff (1973). A value of 2 nM for nisoxetine concentration and a value of 13.8 nM for dissociation constant (K_d) for binding of nisoxetine were employed in these calculations. Values are means ± SE for four determinations.

Table III: Influence of Norepineprhine, Cocaine, and RTI-55 on the Kinetic Parameters, Dissociation Constant (K_d), and Maximal Binding (B_{max}), for Binding of Nisoxetine[a]

compound	K_d (nM)	B_{max} (pmol/mg of protein)
control	13.8 ± 0.4	5.1 ± 0.1
norepinephrine (300 μM)	59.9 ± 4.5	6.6 ± 0.4
cocaine (10 μM)	31.4 ± 1.6	4.9 ± 0.2
RTI-55 (0.1 μM)	26.1 ± 1.2	4.0 ± 0.1

[a] Human placental brush border membranes, suspended in 10 mM Hepes/Tris buffer, pH 7.4, containing 300 mM mannitol, were used in the binding assay. Binding buffer was 50 mM Tris-HCl, pH 7.4, containing 500 mM NaCl. Final concentration of NaCl during binding measurement was 300 mM. Binding was assayed over a nisoxetine concentration range of 5–40 nM (concentration of [^{3}H]nisoxetine was 5 nM and the remaining was unlabeled nisoxetine). Experiments were done in the absence (control) or presence of 300 μM norepinephrine, 10 μM cocaine, or 0.1 μM RTI-55. Assays were carried out at room temperature with a 30-min incubation. Nonspecific binding was determined by performing the binding assay in the presence of 10 μM unlabeled nisoxetine. Kinetic constants, K_d and B_{max}, were determined by Scatchard analysis of the experimental data. Values are means ± SE for 4–16 determinations.

Table IV: Influence of pH on the Kinetic Parameters for Nisoxetine Binding with Respect to the Dependence of Binding on the Concentration of Na^+, Cl^-, and Nisoxetine[a]

kinetic parameter	pH 7.4	pH 5.5
dependence on [Na^+]		
$K_{0.5}$ (mM)	251 ± 12	649 ± 28
n	2.08 ± 0.06	2.20 ± 0.14
dependence on [Cl^-]		
$K_{0.5}$ (mM)	68 ± 2	875 ± 91
n	0.90 ± 0.01	1.04 ± 0.07
dependence on [nisoxetine]		
K_d (nM)	13.8 ± 0.4	51.0 ± 6.7
B_{max} (pmol/mg)	5.1 ± 0.1	6.5 ± 0.6

[a] Human placental brush border membranes, suspended in either 10 mM Hepes/Tris buffer, pH 7.4, containing 300 mM mannitol or in 10 mM Mes/Tris buffer, pH 5.5, containing 300 mM mannitol, were used in the binding assay. Except for the change in the pH of the binding assay, the experimental protocols for the anlysis of the dependence of nisoxetine binding on [Na^+], [Cl^-], and [nisoxetine] were the same as described in the legends to Figures 2–4, respectively. $K_{0.5}$ is the concentration of Na^+ or Cl^- necessary for half-maximal binding, n is the Na^+/nisoxetine or Cl^-/nisoxetine coupling ratio, K_d is the dissociation constant, and B_{max} is the maximal binding capacity. Values are means ± SE for 4–16 determinations.

in the pH range of 7.0–8.5, with half-maximal binding occurring at pH 5.7.

Influence of pH on the Kinetic Parameters for Nisoxetine Binding. We compared the kinetic parameters for binding, determined at two different pH values, 7.4 and 5.5, with respect to the dependence of binding on the concentration of Na^+, Cl^-, and nisoxetine (Table IV). The Na^+/nisoxetine and Cl^-/nisoxetine coupling ratios remained unaltered (2 and 1, respectively) at both pH values as did the maximal binding capacity. However, the affinities of the transporter for Na^+, Cl^-, and nisoxetine were decreased dramatically by changing the pH from 7.4 to 5.5.

Influence of K^+ on Nisoxetine Binding. The presence of K^+ during the binding assay stimulated the Na^+- and Cl^--dependent nisoxetine binding to a small but significant extent. The stimulation was 17 ± 2% at 50 mM K^+ and 27 ± 1% at 100 mM K^+.

Effects of Zn^{2+} and Cyclo(His-Pro) on Nisoxetine Binding. A recent study (Richfield, 1993) has demonstrated that Zn^{2+}, at micromolar concentrations, exerts profound effects on the binding of ligands to the neuronal dopamine transporter. At 10 μM, Zn^{2+} increased the binding of GBR 12935 to the dopamine transporter by 215%. To determine whether Zn^{2+} exerts similar effects on the binding of nisoxetine to the placental norepinephrine transporter, we assayed nisoxetine binding to placental brush border membranes in the presence of varying concentrations of Zn^{2+} (0–1000 μM). Zn^{2+} at concentrations ≤100 μM did stimulate nisoxetine binding, but the effect was much smaller than the stimulatory effect observed in the case of the dopamine transporter. The stimulation of nisoxetine binding was 21 ± 1% at 100 μM Zn^{2+}. However, when the concentration of Zn^{2+} was increased to 1 mM, Zn^{2+} caused a significant inhibition of nisoxetine binding (28 ± 1%). These effects were qualitatively similar to the effects observed with the dopamine transporter (Richfield, 1993) where ligand binding was stimulated by Zn^{2+} at ≤100 μM but inhibited at >100 μM.

Cyclo (His-Pro), a bioactive cyclic peptide (Prasad, 1989), has been recently shown to interact with the neuronal dopamine transporter and inhibit the transporter function with a K_i of 0.09 μM (Jikihara, 1993). Therefore, we investigated the influence of this compound on the binding of nisoxetine to the placental norepinephrine transporter. Cyclo(His-Pro), tested at concentrations ≤100 μM, did not have any effect on nisoxetine binding (data not shown).

DISCUSSION

The Na^+- and Cl^--coupled norepinephrine transporter is selectively inhibited by desipramine and nisoxetine (Graefe & Bonisch, 1988). In the past, desipramine has been widely used as the ligand to characterize this transporter, but there are shortcomings associated with this ligand. Desipramine exhibits a high degree of nonspecific binding and, in addition, interacts with sites other than the norepinephrine transporter (Backstrom *et al.*, 1989; Lee & Snyder, 1981; Lee *et al.*, 1982; Rapp *et al.*, 1987; Slater *et al.*, 1982). This has prompted recent studies to investigate the suitability of nisoxetine as a selective ligand for the norepinephrine transporter (Tejani-Butt, 1992; Tejani-Butt *et al.*, 1990), and these studies have indicated that nisoxetine is indeed a better ligand than desipramine. However, all of these studies have been done only in the rat and there is no information available on the characteristics of binding of nisoxetine to the human norepinephrine transporter. The present investigation was undertaken to conduct a systematic and detailed analysis of the interaction between nisoxetine and the human norepinephrine transporter. We have used in this investigation human placental brush border membranes as the source of the norepinephrine transporter. Our earlier studies have shown that these membranes possess the serotonin transporter (Balkovetz *et al.*, 1989) and the norepinephrine transporter (Ramamoorthy *et al.*, 1992, 1993). The dopamine transporter is absent in these membranes (Ramamoorthy *et al.*, 1993). Since there is a considerable overlap in the specificity between the inhibitors of the dopamine and norepinephrine transporters, the absence of the dopamine transporter in the placental brush border membranes makes these membranes highly suitable for investigations involving the norepinephrine transporter. Furthermore, normal term human placentas are readily obtainable, and large quantities of highly purified brush border membranes can be isolated with ease from this tissue. This makes the placental brush border membranes an excellent experimental tool for detailed analysis of the norepinephrine transporter.

The results described here demonstrate that nisoxetine is a specific, high-affinity ligand for the human norepinephrine transporter. It binds to a single class of binding sites in the placental brush border membrane and competition experiments reveal that this binding site is identical with, or closely related to, the substrate-binding site of the norepinephrine transporter. This conclusion is further supported by the ability of both norepinephrine and nisoxetine to protect the transporter from phenylarsine oxide-induced inhibition, because these experiments reveal that the reactive dithiol groups are located at the binding sites for these ligands and thus suggest the presence of significant similarity in the chemical nature of these binding sites. It is likely that these sites are either identical or overlap considerably with each other. Comparison between the interaction of nisoxetine with the rat norepinephrine transporter (Tejani-Butt, 1992; Tejani-Butt *et al.*, 1990) and the human norepinephrine transporter (this study) indicates several interesting differences as well as similarities. The affinity of the human norepinephrine transporter for nisoxetine is at least an order of magnitude lower than that of the rat norepinephrine transporter (K_d values are 13.8 ± 0.4 nM and 0.8 ± 0.1 nM, respectively). In both cases however, >90% of the binding is due to specific binding. With the rat norepinephrine transporter, the relationship between the

binding and Na^+ concentration is linear even up to 300 mM Na^+, making it impossible to determine the Na^+/nisoxetine coupling ratio (Tejani-Butt, 1992). In contrast, the relationship is sigmoidal in the case of the human norepinephrine transporter, giving a value of 2 for the Na^+/nisoxetine coupling ratio. The dependence of nisoxetine binding on Cl^- has not been investigated in the rat. The present study shows that the binding of nisoxetine to the human norepinephrine transporter has an absolute requirement not only for Na^+ but also for Cl^-. The relationship between the binding and Cl^- concentration is hyperbolic, suggesting a Cl^-/nisoxetine coupling ratio of 1. In addition, the present study provides for the first time a detailed analysis of the role of Na^+ and Cl^- in the binding. Both ions enhance the binding by increasing the affinity of the binding site for the ligand. The maximal binding capacity is not influenced by these ions. The present study also describes for the first time the influence of cocaine and norepinephrine on nisoxetine binding. In the rat, cocaine and norepinephrine when tested up to a concentration of 1 μM, do not inhibit the binding (Tejani-Butt, 1992). This is also true in the case of the human norepinephrine transporter. But both compounds inhibit the binding at higher concentrations, with K_i values of 8.4 ± 0.6 μM and 254.2 ± 17.2 μM, respectively. The inhibition is competitive in nature in both instances. RTI-55, which is an analog of cocaine (Boja *et al.*, 1991), is about 150 times more potent than cocaine in inhibiting the binding of nisoxetine to the human norepinephrine transporter.

The human norepinephrine transporter has been recently cloned from SK-N-SH neuroblastoma cells (Pacholczyk *et al.*, 1991), and Northern blot analysis with the cloned norepinephrine cDNA as the probe has revealed that the human placenta contains transcripts which hybridize to the probe under high stringency conditions (Ramamoorthy *et al.*, 1993). It is very likely that the placental norepinephrine transporter and the neuronal norepinephrine transporter are the products of the same gene. Pacholczyk *et al.* (1991) have determined the inhibitor specificity of the cloned norepinephrine transporter. With respect to the inhibitors which were used in their study as well as in our present study, there is a significant similarity in the relative potency of these compounds to inhibit the cloned transporter and the placental transporter. The order of potency in the case of the cloned transporter is desipramine > nomifensine > imipramine > GBR 12909 > cocaine > paroxetine > serotonin. In the case of the placental transporter, the order of potency is desipramine > nomifensine > imipramine > paroxetine > cocaine > GBR 12909 > serotonin. The small difference observed in the order is most likely due to differences in the assay of the transporter function employed in these two studies. In the study by Pacholczyk *et al.* (1991), the function of the transporter was assayed by determining the 15-min uptake of norepinephrine in intact HeLa cells transfected with the transporter cDNA. In contrast, the function of the transporter was assayed in the present study not by determining the transport parameter but by determining the equilibrium binding of a nontransportable ligand.

There are several similarities between the binding of nisoxetine and desipramine to the norepinephrine transporter. Like the nisoxetine binding, desipramine binding is also Na^+ and Cl^- dependent (Lee & Snyder, 1981; Lee *et al.*, 1982; Bonisch & Harder, 1986; Schomig & Bonisch, 1986). The value for the coupling ratio of Na^+/desipramine is 2 or 3 and the value for the Cl^-/desipramine ratio is 1 (Schomig & Bonisch, 1986). Both Na^+ and Cl^- enhance the binding of desipramine by increasing the binding affinity (Bonisch & Harder, 1986; Zeitner & Graefe, 1986; Ungell *et al.*, 1989). The Na^+:nisoxetine/desipramine stoichiometry of 2:1 observed for the ligand binding is different from the Na^+:norepinephrine stoichiometry of 1:1 observed for transport of norepinephrine (Friedrich & Bonisch, 1986; Ramamoorthy *et al.*, 1993). Similar findings have been reported for the serotonin transporter where apparently one Na^+ ion is involved in serotonin transport (Cool *et al.*, 1990c; Talvenheimo *et al.*, 1983) but two Na^+ ions are involved in the binding of imipramine (Talvenheimo *et al.*, 1983).

Comparison between the potencies of desipramine and cocaine to inhibit norepinephrine transport (Ramamoorthy *et al.*, 1993) and nisoxetine binding (this study) reveals an interesting phenomenon. Desipramine inhibits the transport function and the ligand binding with K_i values of 0.15 and 0.011 μM, respectively. Thus, the ability of desipramine to inhibit the ligand binding is at least an order of magnitude greater than its ability to inhibit the transport. In contrast, cocaine inhibits the transport function and the ligand binding with K_i values of 0.8 and 8.4 μM, respectively. This means that the ability of cocaine to inhibit the ligand binding is an order of magnitude less than its ability to inhibit the transport. Thus, when the assayed function of the norepinephrine transporter is switched from the transport to the ligand binding, the inhibitory potency of desipramine increases, whereas the inhibitory potency of cocaine decreases. The most likely reason for this phenomenon is the differential effects of Na^+ and Cl^- on the interaction of these inhibitors with the transporter. It has been demonstrated that while Na^+ and Cl^- increase the inhibitory potency of desipramine, these ions decrease the inhibitory potency of cocaine (Zeitner & Graefe, 1986; Ungell *et al.*, 1989). In our studies, the transport of norepinephrine was measured in the presence of 112 mM NaCl, whereas the binding of nisoxetine was measured in the presence of 300 mM NaCl. This suggests that, as has been observed in the case of the rat norepinephrine transporter, Na^+ and Cl^- exert differential effects on the interaction of desipramine and cocaine with the human norepinephrine transporter.

The results presented in this report on the effects of H^+ and K^+ on nisoxetine binding are of significance because these ions have profound influence on the transport function of the norepinephrine transporter (Ramamoorthy *et al.*, 1992, 1993). Presence of H^+ and K^+ on the *trans*-side stimulates the NaCl-dependent norepinephrine transport in placental brush border membrane vesicles. The present study shows that H^+ inhibits the binding of nisoxetine to the placental norepinephrine transporter, whereas K^+ stimulates it. However, since these effects are on the equilibrium ligand binding, it is not known whether the observed effects are due to the influence of these ions acting from the *cis*-side or *trans*-side. Interestingly, H^+ does not affect the Na^+/nisoxetine and Cl^-/nisoxetine coupling ratios nor does it influence the maximal nisoxetine binding capacity but reduces the affinities of the transporter for Na^+ and Cl^- as well as nisoxetine.

The pK_a for nisoxetine binding is approximately 5.7. We have no information on the pK_a value for nisoxetine. However, since the titratable group in nisoxetine is a secondary amine ($-NH-CH_3$), the pK_a for nisoxetine is expected to be much higher than 5.7. Therefore, the pK_a value obtained for nisoxetine binding most likely represents the titratability of an amino acid residue in the transporter molecule responsible for the binding of nisoxetine rather than that of the ligand itself.

Because of the reasons that the placental brush border membranes do not have the dopamine transporter (Ramamoorthy *et al.*, 1992, 1993) and that nisoxetine is approximately 1000-fold more effective in inhibiting the function of the norepinephrine transporter than that of the serotonin transporter (Wong & Bymaster, 1976; Wong *et al.*, 1982), the results presented here clearly represent the interaction of nisoxetine with the norepinephrine transporter. The observation that cyclo(His-Pro) does not have any effect on the binding of nisoxetine to the placental brush border membranes and that the stimulatory effect of Zn^{2+} on the binding is at best only minimal show that, unlike nomifensine and mazindol which interact with the dopamine as well as norepinephrine transporters (Javitch *et al.*, 1984; Dubocovich & Zahniser, 1985), nisoxetine is a selective ligand for the norepinephrine transporter.

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